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IGIF Does Not Drive Th1 Development but Synergizes with IL-12 for Interferon-γ Production and Activates IRAK and NFκB

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Summary

In these studies, IFNγ-inducing factor (IGIF), unlike IL-12, did not drive Th1 development in BALB/c or C57BL/6 mice, but like IL-1α, potentiated IL-12–driven Th1 development in BALB/c mice. IGIF and IL-12 synergized for IFNγ production from Th1 cells. Unlike IL-1α, IGIF had no effect on Th2 cells. IGIF signaled through IRAK, IL-1 receptor–associated kinase, to induce nuclear translocation of p65/p50 NFκB in Th1 cells. IL-1α had no effect on proliferation, cytokine production, or NFκB activation in Th1 cells but activated NFκB and proliferation in Th2 cells. Thus, Th1 and Th2 cells may differ in responsiveness and receptor expression for IL-1 family molecules. IGIF and IL-1α may differentially amplify Th1 and Th2 effector responses, respectively.

Introduction

$CD4^+$ T helper (Th) cells can be divided into Th1 and Th2 subsets on the basis of their cytokine profile upon antigen stimulation (Mosmann et al., 1986; Sher and Coffman, 1992; Abbas et al., 1996). The Th1 subset produces interferon-γ (IFNγ) and mediates delayed-type hypersensitivity and protection against intracellular pathogens, while the Th2 subset produces interleukin-4 (IL-4) and IL-5 and is implicated in humoral and allergic responses (Mosmann et al., 1986; Romagnani, 1991; Sher and Coffman, 1992; Abbas et al., 1996). Inappropriate Th subset development can result in failure to clear pathogens, as exemplified by the Th2 response of BALB/c mice to the intracellular organism Leishmania major, and may contribute to immunopathology in human diseases such as atopic asthma and certain autoimmune disorders (Locksley and Scott, 1991; Romagnani, 1991; Sher and Coffman, 1992). Understanding both the factors determining development of Th1 or Th2 responses to antigenic stimulation and the regulation of cytokine production from differentiated Th subsets may thus lead to beneficial manipulation of the immune response.

Cytokines present at the initiation of $CD4^+$ T cell responses determine whether a Th1 or Th2 T cell response predominates (Swain et al., 1991; O'Garra and Murphy, 1994; Paul and Seder, 1994; Romagnani, 1994; Abbas et al., 1996). We and others have shown that IL-12 directs Th1 development from antigen-stimulated naive $CD4^+$ T cell receptor (TCR) αβ transgenic T cells (Hsieh et al., 1993; Manetti et al., 1993; Seder et al., 1993). IL-12 activates signal transducer and activator of transcription-3 (STAT3) and STAT4 in Th1 cells (Jacobson et al., 1995; Szabo et al., 1995), and STAT4 is required for Th1 responses in vivo (Kaplan et al., 1996; Thierfelder et al., 1996). Responsiveness to IL-12 is impaired in BALB/c mice (Hsieh et al., 1995; Guler et al., 1996), which results in the nonhealing Th2 response to L. major infection (Scott et al., 1988; Heinzel et al., 1989).

IFNγ-inducing factor (IGIF) was discovered in studies of IFNγ production in a Proprionobacterium acnes–induced model of toxic shock (Okamura et al., 1995). This cytokine was subsequently characterized as active in promoting proliferation and IFNγ production by Th1 clones and lines and natural killer (NK) cells in both mice and humans and was suggested to have potency similar to that of IL-12 (Okamura et al., 1995; Micallef et al., 1996; Ushio et al., 1996; Kohno et al., 1997). However, the demonstration that Th1 responses were defective in mice with a disrupted IL-12 gene confirmed a central role of IL-12 in Th1 development (Magram et al., 1996). The role of IGIF in Th1 development has not yet been assessed. Structural analysis and fold recognition suggest that IGIF belongs to the IL-1 family (Bazan et al., 1996). This hypothesis is supported by the observation that IGIF is synthesized as an inactive precursor that requires cleavage by caspase 1 for activity (Ghayur et al., 1997; Gu et al., 1997). We have recently demonstrated that IL-1α acts as a cofactor in IL-12–induced Th1 development in BALB/c but not C57BL/6 mice, and that IL-1α responsiveness is lost by committed Th1 cells and clones (K. S. et al., submitted), in agreement with previous reports of loss of IL-1α binding by Th1 clones (Lichtman et al., 1988). IL-1α signaling takes place via IL-1 receptor-associated kinase (IRAK), which activates a cascade through NIK and CHUK kinases, leading to activation of nuclear factor κB (NFκB) (Cao et al., 1996; Malinin et al., 1997; Regnier et al., 1997).

In this study we show that IGIF, unlike IL-12, does not drive Th1 development but potentiates IL-12–induced Th1 development in BALB/c but not C57BL/6 mice. Furthermore, we show marked synergy between IGIF and IL-12 in inducing IFNγ production from differentiating and committed Th1 cells from BALB/c and C57BL/6 mice, suggesting that both IL-12 and IGIF are required for significant expression of the Th1 phenotype. Unlike IL-12, IGIF does not activate STAT4 in Th1 cells, but rather signals through the IRAK pathway to induce nuclear translocation of p65/p50 NFκB complex. In contrast, IL-1α, which showed no effect on Th1 cells, activated NFκB and induced proliferation of Th2 cells, which did not respond to IGIF. Th1 and Th2 cells thus differ in responsiveness and receptor expression for IL-1 family molecules, and IGIF and IL-1α may differentially amplify Th1 and Th2 effector responses respectively.

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Results

IGIF Does Not Drive Th1 Development but Potentiates IL-12–Induced Th1 Development

In initial studies, IGIF was described as a factor that could act independently of IL-12 in promoting IFNγ production from Th1 cells and suggested to be as potent as IL-12 (Okamura et al., 1995; Micallef et al., 1996; Kohno et al., 1997). Although IL-12 has been shown to drive Th1 development (Hsieh et al., 1993; Macatonia et al., 1993; Manetti et al., 1993; Seder et al., 1993), the role of IGIF in Th1 development has not been assessed. We therefore examined the effect of addition of IGIF, alone or with IL-12, in inducing Th1 development from naive D011.10 ovalbumin-specific TCR transgenic $CD4^+$ T cells (Murphy et al., 1990; Hsieh et al., 1993; Macatonia et al., 1993) cultured for 7 days with irradiated dendritic cells and ovalbumin peptide 323–339 ($OVA_{323–339}$). T cells were restimulated with dendritic cells or spleen antigen-presenting cells (APC) and OVA, and IFNγ was measured in culture supernatants at 48 hr by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 1A, addition of IGIF to primary cultures of naive $CD4^+$ T cells stimulated with dendritic cells did not itself induce Th1 development. However, IGIF potentiated IL-12–induced Th1 development.

IGIF Potentiates IL-12–Induced Th1 Development in BALB/c but Not C57BL/6 Mice

BALB/c mice preferentially develop IL-4 predominant immune responses to L. major infection (Locksley and Scott, 1991) and in vitro (Hsieh et al., 1995; Guler et al., 1996). Our previous studies have shown that IL-1α is a cofactor for IL-12–induced Th1 development in BALB/c but not C57BL/6 mice (K. S. et al., submitted). Therefore, the role of IGIF in Th1 development in C57BL/6 versus BALB/c $CD4^+$ T cells was examined. Naive $CD4^+$ T cells from BALB/c and C57BL/6 mice were stimulated with plate-bound anti-CD3 and IL-2 (to exclude any role of APC-derived factors) for 7 days in the presence or absence of IGIF and/or IL-12. The cells were restimulated with anti-CD3 and IL-2, and IFNγ production was assessed at 48 hr. IGIF alone did not induce Th1 development in either mouse strain (Figure 1B). However, potentiation of IL-12–driven Th1 development by IGIF was observed in this APC-free system in BALB/c but not C57BL/6 mice (Figure 1B). These results suggest that IGIF acts directly on the T cell and that there is a strain difference in T cell IL-12 responsiveness.

Szabo et al. (1997) have recently defined the molecular basis of differential IL-12 responsiveness of murine Th subsets by demonstrating the reciprocal regulation of the IL-12Rβ2 subunit by IL-4 and IFNγ. IFNγ up-regulates the IL-12Rβ2 chain and counteracts the inhibitory effects of IL-4. Thus BALB/c mice, which produce a substantial amount of IL-4 (Hsieh et al., 1995; Guler et al., 1996), may down-regulate IL-12Rβ2 expression, imposing the reported requirement for IFNγ in Th1 development. This raises the possibility that IGIF acts either to down-regulate IL-4 or to up-regulate IFNγ directly, thus increasing the IL-12 responsiveness of BALB/c T

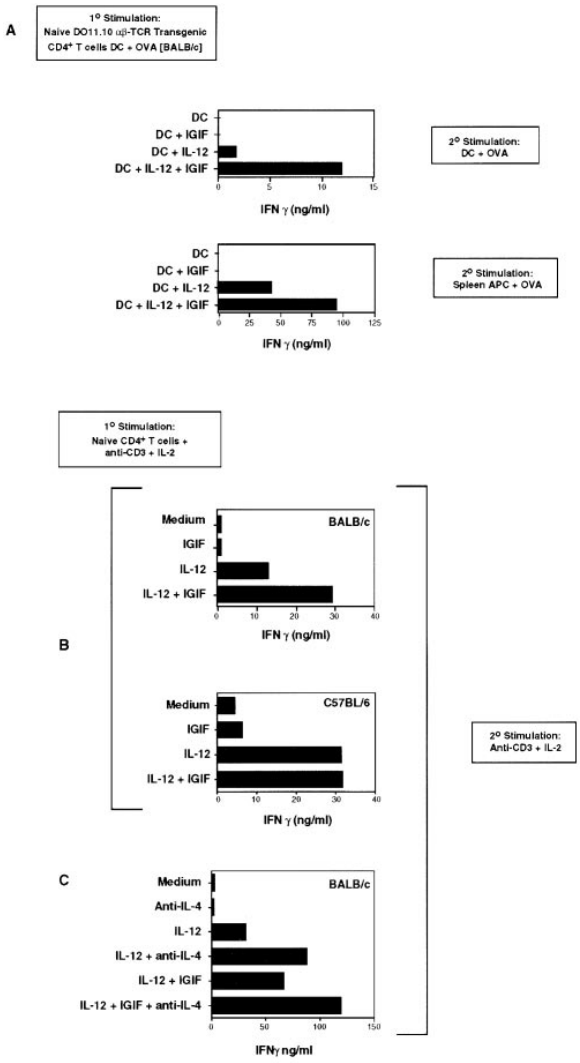

Figure 1. IGIF Does Not Drive Th1 Development, but Potentiates IL-12–Driven Th1 Development in BALB/c but not C57BL/6 Mice

(A) IGIF does not drive Th1 development but synergizes with IL-12 in Th1 development in BALB/c mice. Naive $CD4^+$ T cells from DO11.10 TCR transgenic mice were cultured in 24 well plates at a density of 2.5×10^5 T cells in 2 ml with irradiated FACS-purified dendritic cells (1×10^4/well) and antigen for 7 days in the presence of medium alone, IGIF, IL-12, or IGIF plus IL-12. Cells were then restimulated at 2.5×10^5/ml with antigen presented either by dendritic cells (DC) or spleen APC (5×10^6/well) for 48 hr. IFNγ was measured in 48 hr supernatants by ELISA. Results are representative of at least five independent experiments.

(B) IGIF potentiates IL-12–induced Th1 development from BALB/c T cells, but not C57BL/6 T cells in an APC-free development culture. Naive $CD4^+$ T cells from BALB/c or C57BL/6 mice (5×10^5/well) were cultured for 7 days in 24-well plates coated with anti-CD3 with either IL-2 alone (medium) or in combination with IGIF, IL-12, or both IGIF and IL-12. Cells were restimulated with anti-CD3 and IL-2 for 48 hr.

(C) IGIF potentiation of IL-12–induced Th1 development from naive BALB/c T cells results in part from antagonism of the effects of endogenous IL-4. Naive BALB/c $CD4^+$ T cells were cultured for 7 days with anti-CD3 and IL-2, in the presence of anti-IL-4 (11B11, 10 μg/ml), IL-12, IL-12 plus anti-IL-4, IGIF plus IL-12, or IGIF plus IL-12 and anti-IL-4. Cells were then restimulated with anti-CD3 and IL-2 for 48 hr. Results are representative of three independent experiments.

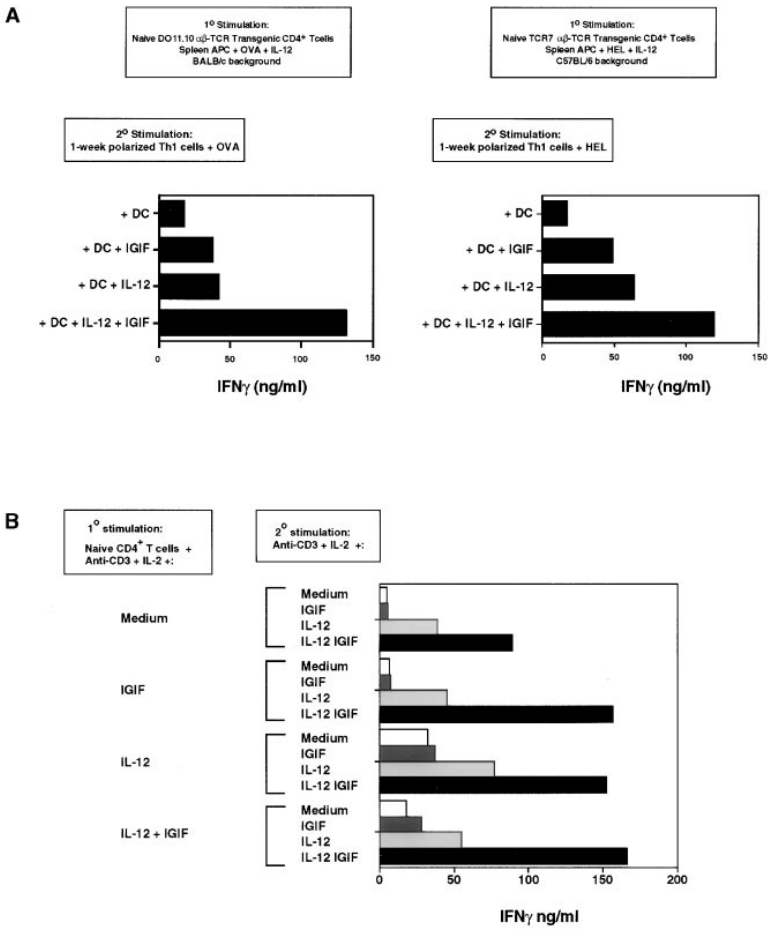

Figure 2. IGIF Synergizes with IL-12 to Increase IFNγ Production from Differentiating Th1 Cells

(A) IGIF synergizes with IL-12 in secondary cultures to increase IFNγ production from differentiating Th1 cells from both BALB/c and C57BL/6 mice when dendritic cells (DC) are used as APC for restimulation. Naive $CD4^+$ T cells from D011.10 (BALB/c) or TCR7 (C57BL/6) TCR transgenic mice were cultured with antigen and whole spleen cells for 7 days. Cells (2.5×10^5 /well) were restimulated with antigen presented by irradiated dendritic cells (1×10^4/well) in medium alone or with IGIF, IL-12, or IGIF plus IL-12 in the secondary culture for 48 hr; then supernatants were harvested and IFNγ measured by ELISA. Results are representative of five independent experiments.

(B) IGIF synergizes with IL-12 during restimulation of differentiating Th1 cells to reveal Th1 development even in neutral conditions. Naive T cells from C57BL/6 mice were cultured in anti-CD3-coated 24-well plates (5×10^5/well) with IL-2 (10 ng/ml) and medium alone, IGIF, IL-12, or IGIF plus IL-12 for 7 days. Cells were then restimulated with anti-CD3 and IL-2 plus medium alone, IGIF, IL-12, or IGIF plus IL-12 for 48 hr. IFNγ was measured in supernatants by ELISA. Results are typical of three separate experiments.

Similar results were seen with BALB/c T cells (data not shown).

cells. To test this hypothesis, anti-IL-4 antibodies were added during culture of naive BALB/c $CD4^+$ T cells with anti-CD3 and IL-2 in the presence or absence of IGIF and IL-12. As shown in Figure 1C, anti-IL-4 antibodies augmented IL-12–driven Th1 development from BALB/c T cells and partially counteracted the requirement of IGIF. This result may not occur in mice from genetic backgrounds producing low levels of IL-4, such as C57BL/6. Indeed, IGIF was not required to potentiate IL-12–driven Th1 development in C57BL/6 mice (Figure 1C), nor was there an effect with anti-IL-4 antibodies (data not shown).

IGIF Synergizes with IL-12 in Inducing IFNγ Production from 1-Week–Stimulated Th1 Cells

Restimulation of 1 week–polarized Th1 cells with dendritic cells and antigen, or anti-CD3, even in the presence of added IL-12, led to production of significantly lower levels of IFNγ than those observed upon restimulation with splenic APC and antigen (Figure 2A, and K. S. et al., submitted). Th1 cells were derived from TCR transgenic $CD4^+$ T cells from DO11.10 mice (BALB/c) or from hen egg lysozyme peptide 74–88 ($HEL_{74–88}$)–specific TCR7 mice (C57BL/6) (S. B. H. et al., unpublished data) stimulated in the presence of IL-12 for 7 days. To test the effect of IGIF on IFNγ production from these developing Th1 cells, IGIF and IL-12, either alone or in combination, were added to the 48 hr restimulation culture. As shown in Figure 2A, IGIF induced a small increase in IFNγ production when added alone to Th1 cells restimulated with dendritic cells and antigen but showed marked potentiation of IL-12–induced IFNγ production. In contrast to its effect on primary Th1 development, IGIF potentiation of IL-12–induced IFNγ production in secondary cultures was seen when either DO11.10 transgenic T cells (BALB/c) or TCR7 transgenic T cells (C57BL/6) were used.

Marked potentiation of IFNγ production by IL-12 and IGIF was also observed when Th1 cells were stimulated with anti-CD3 in the absence of APC. Again, this synergy occured in both BALB/c (data not shown) and C57BL/6 Th1 cells (Figure 2B). When both IGIF and IL-12 were included in secondary stimulation of T cells previously cultured with anti-CD3 and IL-2 for 1 week in the absence of IL-12, highly significant levels of IFNγ were also produced (Figure 2B). Indeed, the combination of IGIF with IL-12 in the secondary stimulation led to levels of IFNγ production from anti-CD3–stimulated cells similar to those from cells restimulated with antigen and splenic APC. These data, obtained using an APC-free system in which endogenous IL-12 production could be ruled out, suggest that IL-12 is not required for development of the potential for Th1 cells to produce IFNγ. Of importance is that there is an absolute requirement for IL-12 in the induction of IFNγ production from developing Th1 cells, which is dramatically enhanced by its synergy with IGIF.

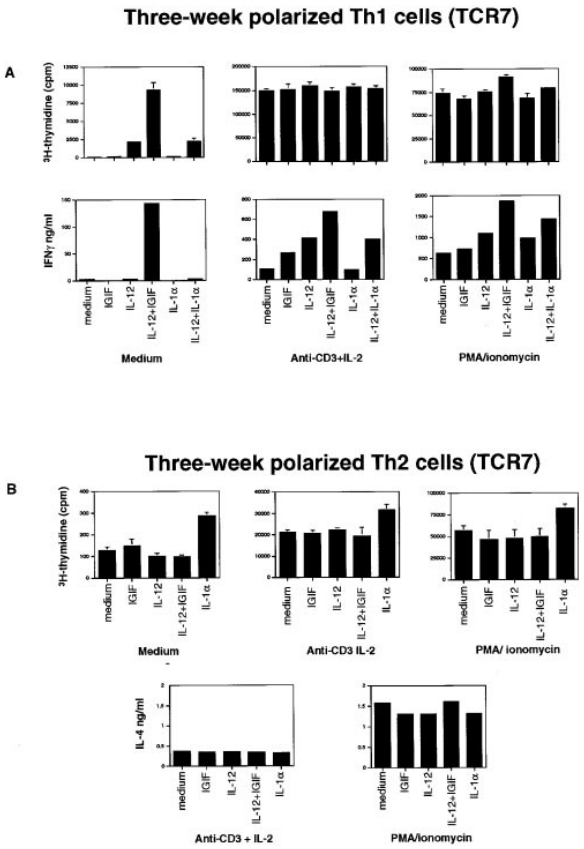

Figure 3. IGIF Potentiates IL-12–Induced Proliferation and IFNγ Production from Committed Th1 Cells but Does Not Affect Th2 Cells, Which Respond to IL-1α

Committed Th1 and Th2 cells were derived from naive $CD4^+$ T cells from TCR7 TCR transgenic mice (C57BL/6 background) stimulated with antigen presented by irradiated splenic APC for three rounds of 1 week in the presence of IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) (A) or IL-4 (10 ng/ml) and anti-IL-12 (10 μg/ml) (B), respectively. Cells were then rested in IL-2 prior to 48 hr culture (at 5×10^4/well) with medium alone, IGIF, IL-12, IGIF plus IL-12, IL-1α, or IL-1α plus IL-12 in 96-well plates. These 48 hr cultures were in the presence of either medium alone, soluble anti-CD3 (100 ng/ml) plus IL-2 (10 ng/ml), or PMA (50 ng/ml) and ionomycin (500 ng/ml). IFNγ or IL-4 production was measured by ELISA, and proliferation was assessed by [^{3}H]thymidine incorporation. Results are representative of three separate experiments, and similar results were seen with cells derived from DO11.10 TCR transgenic animals.

IGIF Synergizes with IL-12 to Stimulate Proliferation and IFNγ Production by Committed Th1 but Not Th2 Cells

Committed Th1 and Th2 populations were derived from DO11.10 and TCR7 TCR transgenic mice by repeated antigenic stimulation of naive $CD4^+$ T cells with splenic APC in the presence of IL-12 and anti-IL-4 antibodies or IL-4 and anti-IL-12 respectively. After three rounds of antigenic stimulation in the presence of polarizing cytokines, these cells acquire a stable, committed Th phenotype (Murphy et al., 1996). Polarized Th1 and Th2 cells and Th1 and Th2 clones (data not shown) were then rested for 10 days in IL-2 prior to 48 hr culture with IGIF, IL-1α, and IL-12, alone or in combination. In these cultures cells were either unstimulated or were stimulated with soluble anti-CD3 and IL-2 or with phorbol myristate acetate (PMA) and ionomycin, and were tested for IFNγ and IL-4 production and proliferation at 48 hr. As shown in Figure 3A, IGIF alone had a minimal effect on proliferation of polarized Th1 cells. Additive or synergistic effects of IGIF and IL-12 on IFNγ production and proliferation were seen with both anti-CD3 and PMA and ionomycin stimulation (Figure 3A). IGIF also acted with IL-12 to potentiate proliferation and IFNγ production from Th1 clones in response to stimulation with anti-CD3 and IL-2, or PMA/ionomycin, in agreement with previous findings (Kohno et al., 1997) (Figure 3A). Of note, maximal synergy between IL-12 and IGIF was seen in conditions of suboptimal TCR stimulation, such as by soluble anti-CD3 and IL-2, and even without TCR engagement (Figure 3A). In contrast, IL-1α had no significant effect on proliferation or IFNγ production by polarized Th1 cells or Th1 clones (data not shown), either alone or in combination with IL-12 (Figure 3A).

Previous reports show that IL-1α acts to support proliferation and IL-4 responsiveness of Th2 clones (Greenbaum et al., 1988; Lichtman et al., 1988; Weaver et al., 1988; McArthur and Raulet, 1993). In contrast to Th1 cells, IL-1α supported proliferation of polarized Th2 cells and Th2 clones (data not shown), whereas IGIF, alone or in combination with IL-12 or IL-4, had no effect on proliferation or IL-4 production by polarized Th2 cells (Figure 3B). IGIF, alone or in combination with IL-12, did not induce IFNγ production from committed Th2 cells or clones (data not shown).

IGIF Does Not Induce STAT4 Activation in Th1 Cells

IL-12 activates both STAT3 and STAT4 in Th1 cells (Jacobson et al., 1995; Szabo et al., 1995), and STAT4 is required for Th1 responses (Kaplan et al., 1996; Thierfelder et al., 1996). Our results suggest that IGIF cannot replace IL-12 but acts to potentiate IL-12–induced IFNγ production. We examined the ability of IGIF to induce STAT4 activation in committed Th1 cells and clones. Committed Th1 cells or Th1 clones, were rested in IL-2 for 10 days. After a further 5 hr rest in medium with reduced serum content (2% fetal calf serum [FCS]), cells were cultured for 30 min in medium alone, IL-12, IGIF, or the combination of both cytokines. Nuclear lysates were then prepared and run in electromobility gel shift assays (EMSA) with a ^{32}P-labeled m67SIE nucleotide probe to detect STAT binding. To distinguish STAT4 from other STATs, supershifting was performed by preincubation of the lysate with an anti-STAT4 antibody, NB34 (Guler et al., 1997). As shown in Figure 4, SIE binding protein was detected in nuclear lysates from Th1 cells stimulated with IL-12, and it was confirmed to be STAT4 by supershifting with an antibody to STAT4. No induction of SIE binding activity was observed in nuclear lysates from cells stimulated with IGIF. These results show that IGIF, unlike IL-12, does not induce STAT4 activation in committed Th1 cells from either BALB/c or C57BL/6 mice or from Th1 clones (Figure 4). Since the m67SIE probe detects STAT1 and STAT3 in addition to STAT4 (Szabo et al., 1997), IGIF does not appear to induce any of the STATs shown to act in Th1 development and phenotype expression. Furthermore,

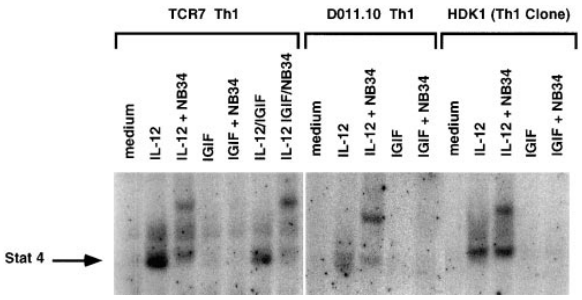

Figure 4. IGIF Does Not Activate STAT4

Nuclear lysates were prepared from committed Th1 cells from TCR7 and DO11.10 TCR transgenic mice or from HDK 1 Th1 clones after incubation in medium, IL-12, IGIF or IL-12 plus IGIF for 30 min. Lysates (2 μg of protein) were incubated for 15 min with either anti-STAT4 antibody (NB34) or phosphate-buffered saline at room temperature and then with ^{32}P-labeled m67SIE double-stranded oligonucleotide probe. Complexes were run on 5.25% polyacrylamide gels in 0.25× Tris-borate-EDTA. Bands were seen when samples were incubated with IL-12 but not IGIF, and supershift with anti-STAT4 antibody confirmed these bands as including STAT4 complexes. Addition of IGIF did appear to influence bands seen with IL-12. Similar results were seen in three separate experiments.

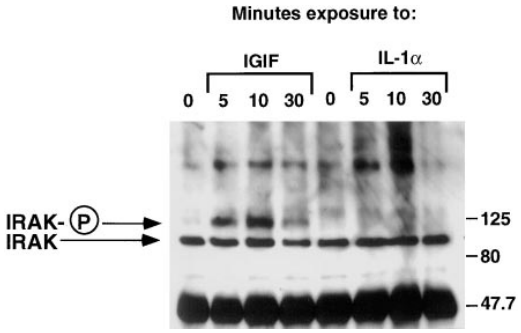

Figure 5. IGIF, but Not IL-1α, Activates IRAK in Committed Th1 Cells

Committed Th1 cells from DO11.10 TCR transgenic mice (2×10^7 cells) were rested in 2% FCS-containing medium for 5 hr and then in medium containing IGIF or IL-1α for 0, 5, 10, and 30 min. Whole-cell lysates were prepared and IRAK was immunoprecipitated with a rabbit antiserum. Antibody complexes were separated by protein A beads and then run on an SDS polyacrylamide gel and transfered to a membrane for Western blotting with the antiserum to IRAK. Bound antibody was detected with anti-rabbit–horseradish peroxidase complex and visualized with chemiluminescent substrate. After 5, 10, and 30 min in IGIF, an additional, higher molecular weight band was seen, reflecting an increased molecular mass of IRAK, presumably by autophosphorylation (IRAK-P). No such size increase was seen with IL-1α. Similar results were seen in three experiments and with the HDK1 Th1 clone.

IGIF did not appear to increase IL-12–induced STAT4 activation.

IGIF Induces Activation of IL-1 Receptor–Associated Kinase (IRAK)and Nuclear Translocation of p65/p50 NFκB in Th1 Cells

IL-1α/β binds to IL-1 receptors and activates a signaling pathway that involves IL-1 receptor–associated kinase (IRAK) and ultimately leads to nuclear translocation of the transcription factor NFκB (Cao et al., 1996; Malinin et al., 1997; Regnier et al., 1997). To test the possible participation of IRAK in IGIF-induced signaling, whole-cell lysates were prepared from committed Th1 cells from DO11.10 mice stimulated with either IGIF or IL-1α for 0, 5, 10, and 30 min. Lysates were incubated with antiserum to IRAK (Cao et al., 1996); immunoprecipitated protein was then run on Laemmli gels and detected with the same antiserum. As shown in Figure 5, IGIF induced a time-dependent appearance of IRAK bands with a higher molecular mass in Th1 cells, and this was not seen with IL-1α. Similar results were seen in Th1 clones (data not shown). This result suggests that IGIF signals through a receptor that associates with IRAK but is distinct from the IL-1 receptor type 1, since IL-1α does not act on these cells. This finding is in agreement with the observation that IGIF activity on NK cells is not blocked by antibodies to either IL-1R1, IL-1R2, or the IL-1 accessory receptor (Hunter et al., 1997), suggesting that IGIF uses a novel IL-1R. Several orphan IL-1R molecules exist (Hardiman et al., 1996).

We next examined nuclear lysates from Th1 cells, stimulated with IGIF, IL-12, IL-1α, or PMA and ionomycin for 30 min, for binding activity to a ^{32}P-labeled κB nucleotide probe. Inducible NFκB binding was seen both in committed Th1 cells (Figure 6A) and in Th1 clones (Figure 6B) incubated with IGIF (the higher molecular weight band), whereas only a single constitutive band was seen in unstimulated cells and in cells stimulated with IL-1α or IL-12. Competition with unlabeled probe confirmed the specificity of both the constitutive and IGIF-induced bands (Figure 6C). Supershifting with a panel of antibodies to various Rel family members showed that the induced band was supershifted with both anti-p65 and anti-p50 antibodies, whereas the constitutive band shifted only with anti-p50 (Figure 6). This indicates that a constitutive p50/p50 homodimer is expressed in the nucleus of unstimulated Th1 cells and that IGIF induced nuclear translocation of p65/p50 heterodimers. IGIF also induced nuclear translocation of p65/p50 NFκB complexes in committed Th1 cells from C57BL/6 mice (data not shown). These data show that, unlike IL-12, IGIF activates NFκB in Th1 cells, suggesting that these factors activate separate signal transduction pathways that may interact to produce the synergistic biological effects observed.

Th2 Cells Do Not Respond to IGIF, but to IL-1α, Which Induces Proliferation and Nuclear Translocation of NFκB

To address whether differences in responsiveness between Th1 and Th2 cells resulted from differential activation of NFκB signaling in the two subsets by IGIF and IL-1α respectively, nuclear lysates from committed Th1 and Th2 cells and Th1 and Th2 clones were analyzed. As shown in Figures 7A and 7B, IGIF, but not IL-1α, caused nuclear translocation of p65/p50 NFκB complexes in Th1 cells but not Th2 cells. In contrast, IL-1α activated p65/p50 NFκB complexes in Th2 cells but not Th1 cells. Taken together, these data suggest that differential responsiveness of Th1 and Th2 cells to the IL-1 family members IGIF and IL-1α may be determined at the level of receptor expression. Moreover, the data

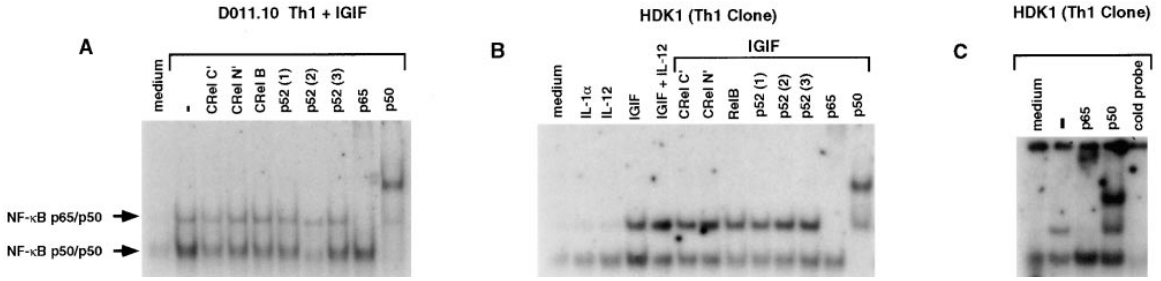

Figure 6. IGIF Induces Nuclear Translocation of p65/p50 NFκB Complexes in Th1 Cells

Nuclear lysates were prepared from DO11.10 Th1 polarized cells (A) and the Th1 clone HDK 1 (B and C) and cultured for 30 min with medium alone, IL-1α, IL-12 or IGIF. Lysates were incubated with antibodies to Rel family members for 15 min and then with ^{32}P-labeled double-stranded oligonucleotide NFκB probe before they were run on 5.25% polyacrylamide gels in 0.25× Tris-borate-EDTA. A constitutive NFκB complex was seen in Th1 cells, shown to be a p50/p50 homodimer by supershifting, and an additional NFκB complex, a p65/p50 heterodimer, was seen after incubation with IGIF. (C) NFκB complexes were not seen after preincubation of lysates with nonlabeled probe (cold competition), confirming the specificity of the bands observed. Similar results were seen in committed Th1 cells from TCR7 (C57BL/6) TCR transgenic mice and in four separate experiments.

suggest that this difference may lead to differential amplification of Th1 or Th2 responses by these factors produced during innate immune responses.

Discussion

IL-12 knockout mice and STAT4 knockout mice have indicated their critical role in Th1 responses (Kaplan et al., 1996; Magram et al., 1996; Thierfelder et al., 1996). Several cofactors for IL-12–driven Th1 responses have been identified for particular genetic backgrounds, including IL-1α in BALB/c mice (K. S. et al., submitted). The reported structural similarity between IL-1α and IGIF led us to investigate and compare the roles of IGIF and IL-1α in Th1 development and in IFNγ production from committed Th1 cells and clones, including an analysis of the signal transduction pathways. Our studies show that IGIF does not in itself induce Th1 development, but acts with IL-12 as an important amplifying factor for IFNγ production. The signal transduction pathway that is activated by IGIF is the IL-1α pathway and includes activation of IRAK and nuclear translocation of p65/p50 NFκB, but does not involve the IL-1R1 receptor. In contrast, IGIF has no activity in signaling effector functions of Th2 cells, nor does it activate the NFκB pathway. However, Th2 cells respond to another IL-1 protein, IL-1α. The differential responsiveness between Th1 and Th2 cells to IGIF and IL-1α respectively may have profound implications for regulation of ongoing Th cell responses.

The finding that IGIF does not drive Th1 development is consistent with the requirement for IL-12 and STAT4 for Th1 responses (Kaplan et al., 1996; Magram et al., 1996; Mattner et al., 1996; Thierfelder et al., 1996). IGIF, like IL-1α, acted as a cofactor in IL-12–induced Th1 development in BALB/c mice. We have previously shown that IL-1α and TNFα increase IL-12 responsiveness of developing BALB/c Th1 cells (K. S. et al., submitted). Cofactors were not required for Th1 development from CD4$^+$ T cells from C57BL/6 mice. BALB/c mice preferentially mount Th2 responses and IL-4 production (Hsieh et al., 1995). Since IL-4 has been shown to down-regulate the IL-12Rβ2 subunit and hence IL-12 responsiveness (Szabo et al., 1997), it is possible that IGIF acts to reverse the inhibitory effect of IL-4 on IL-12 responsiveness of developing BALB/c Th1 cells. This idea was supported by our finding that anti-IL-4 partially

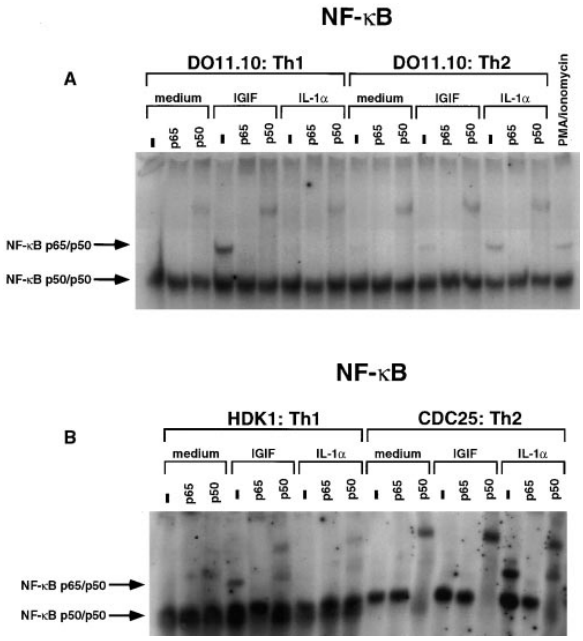

Figure 7. IGIF but Not IL-1α Activates p65/p50 NFκB in Th1 Cells, whereas IL-1α but Not IGIF Activates p65/p50 NFκB in Th2 Cells

Committed Th1 and Th2 cells (A) and Th1 and Th2 clones (B) were incubated with medium, IGIF, or IL-1α for 30 min, and nuclear lysates were then made. Nuclear lysates (2 μg of protein) were incubated with antibodies to p65 (RelA) or p50 NFκB components or saline and then with ^{32}P-labeled double-stranded oligonucleotide NFκB probe, and then were run on a 5.25% polyacrylamide gel. Constitutive p50/p50 complexes were observed in both Th1 and Th2 cells. IGIF but not IL-1α induced p65/p50 complexes in Th1 cells. In contrast, IL-1α but not IGIF did so in Th2 cells. Both p65/p50 and p50/p50 NFκB complexes were seen in Th1 cells stimulated with PMA and ionomycin (A). Results are representative of three separate experiments.

obviated the effect of IGIF on IL-12–induced Th1 development in BALB/c mice. It is possible that differences in levels of IL-4 production during Th development determine in part the requirement for cofactors in IL-12–induced Th1 development. We found that anti-IL-4 antibodies did not influence Th1 development from $CD4^+$ T cells from C57BL/6 mice. Our results may be relevant for improving our understanding of responses to intracellular pathogens. For example, susceptibility or resistance to L. major in various strains of mice is complex and probably is controlled by several genetic loci (Beebe et al., 1997; Roberts et al., 1997). Although this may result from regulation of the level of IL-12Rβ2 expression by IL-4 or IFNγ, it is possible that the immune response is also affected by other cofactors, such as IGIF or IL-1α, and by their interplay with T cells and NK cells (Bancroft et al., 1989; D'Andrea et al., 1992; Scharton and Scott, 1993).

In an APC-free system, IGIF showed marked synergy with IL-12 in inducing IFNγ production by developing Th1 cells from both BALB/c and C57BL/6 mice. Furthermore, the addition of IGIF plus IL-12 in the secondary stimulation of T cells in this system revealed that Th1 development can occur in the absence of IL-12. This IL-12–independent Th1 development was abrogated by antibodies to IFNγ (data not shown), a finding in keeping with previous reports that IFNγ can direct Th1 development through effects on both T cells and APC (Sher and Coffman, 1992; Seder et al., 1993; Dighe et al., 1995; Lohoff et al., 1997; Taki et al., 1997). However, we show that IL-12 is absolutely required for induction of IFNγ production from developing Th1 cells and that this action is markedly augmented by IGIF. This requirement was not previously appreciated because Th1 cells were typically restimulated in cultures with antigen and spleen APC, which may have provided endogenous levels of IGIF, and low levels of IL-12 (Murphy et al., 1994). This effect of IL-12 and IGIF may overcome the inhibition by endogenous factors acting to suppress IFNγ production in differentiating Th1 populations, which are heterogeneous in terms of cytokine production (Openshaw et al., 1995; Murphy et al., 1996).

IGIF had minimal effect alone on inducing IFNγ production from committed Th1 cells or Th1 clones, in contrast to previously reported findings in Th1 cell lines and clones (Kohno et al., 1997). The cells used in our experiments had previously been rested in IL-2–containing media and were used at least 10 days after their last antigenic stimulation to avoid any carry-over effect of antigenic stimulation, which may explain this apparent discrepancy. In agreement with the findings of Kohno et al. (1997), we observed synergy between IGIF and IL-12 in induction of IFNγ production from committed Th1 cells and clones. Indeed, this synergy was observed even in the absence of TCR stimulation or in conditions of suboptimal TCR stimulation. This raises the possibility of considerable amplification of Th1 responses by the combination of IGIF and IL-12 in vivo, and it is possible that bystander activation of previously committed Th1 cells of different antigen specificities might contribute to this amplification. Moreover, such bystander Th1 activation has the potential for harmful activation of memory Th1 cells cross-reactive with self-antigens.

Our findings on signaling by IGIF are in accordance with the observed biology. IGIF, unlike IL-12, did not induce STAT4 activation in Th1 cells and did not drive Th1 development. Since IL-12 and STAT4 gene deletions lead to mice with defective Th1 responses, STAT4 activation appears to be a critical step in the activation of Th1 development (Jacobson et al., 1995; Szabo et al., 1995; Kaplan et al., 1996; Thierfelder et al., 1996). Potential STAT4 response elements have been proposed though not demonstrated in the IFNγ promoter (Young, 1996), and the target genes of STAT4 in Th1 development are not known. We show that, like IL-1α, IGIF signals through IRAK, leading to nuclear translocation of NFκB p65/p50 complexes. A number of NFκB sites have been described in the IFNγ promoter and introns (Young, 1996), so it is likely that IGIF-induced p65/p50 acts directly on the IFNγ promoter, presumably in cooperation with other transcription factors induced via STAT4 activation. However, IGIF signaling and responses were confined to Th1 cells, whereas in agreement with a previous report (Lederer et al., 1996) only Th2 cells showed NFκB activation and proliferation to IL-1α. Thus, although IL-1α and IGIF share signaling machinery, these molecules have widely divergent effects on differentiated Th cell subsets. It has been shown that IL-1α/β receptors are lost on Th1 clones (Lichtman et al., 1988), and IGIF activity on NK cells is not blocked by antibodies to IL-1R1, IL-1R2, or IL-1R3 (Hunter et al., 1997). Differences in the activity of IGIF and IL-1α may therefore be determined at the level of receptor expression. IL-1 receptors and the related Toll receptors activate NFκB (Medzhitov et al., 1997; Regnier et al., 1997), and this pathway is widely conserved across Drosophila melanogaster, mammalian cells, and plants (Wilson et al., 1997). It has an important role in development and is critical in the innate immune response. The actions of IGIF via IRAK and NFκB in Th1 cells and the actions of IL-1α via IRAK and NFκB in Th2 cells may represent an important interface between the innate and acquired immune responses. Thus these IL-1 family members may act on specific limbs of T cell activation, with IGIF acting in concert with IL-12 to amplify Th1 responses and IL-1α/β enhancing Th2 responses.

In summary, IGIF does not by itself drive Th1 development and does not activate STAT4, in contrast to IL-12. However like IL-1α, IGIF potentiates IL-12–driven Th1 development in BALB/c but not C57BL/6 mice. The major effect of IGIF in both BALB/c and C57BL/6 mice is synergy with IL-12 for expression of the Th1 phenotype through IFNγ production. These findings indicate that signals from both IL-12 and IGIF are required to induce significant levels of IFNγ production from resting Th1 effector cells. This is supported by the effects on Th1 responses in vivo, observed either by removal of IL-12 (Magram et al., 1996) or neutralization of IGIF (Okamura et al., 1995). Taken together, these data argue for the relevance of this synergy between IL-12 and IGIF and thus the signals through STAT4 and NFκB in host protection against intracellular pathogens and in possible induction of immunopathology. This raises the possibility that intervention in the actions of either or both IL-12 and IGIF might hold therapeutic potential, either in augmenting protective Th1 responses, as in mycobacterial

disease, or in diminishing damaging Th1 responses, as in autoimmune disease.

Experimental Procedures

Animals

Mice transgenic for an αβ TCR recognizing $OVA_{323–339}$ (D011.10; BALB/c genetic background) (Murphy et al., 1990) were selected at age 4–6 weeks by staining peripheral blood leukocytes with the anti-clonotype monoclonal antibody (MAb) KJ1–26 (Haskins et al., 1983). Mice transgenic for an αβ TCR recognizing a peptide of hen egg lysozyme, $HEL_{74–88}$ (TCR7, C57BL/6 background; S. B. H. et al., unpublished data) were selected at age 4–6 weeks by polymerase chain reaction analysis for the rearranged transgenic TCR. All transgenic mice used in the experiments were heterozygous for the integration of the TCR αβ chains. BALB/c mice 6–10 weeks old were purchased from Simonsen (Gilroy, CA); C57BL/6 mice between 6–10 weeks old were purchased from Jackson (Bar Harbor, ME).

Culture Medium, Cytokines, Antibodies, and Antigens

RPMI 1640 (JR Scientific, Woodland, CA) supplemented with 10% FCS (JR Scientific), 2-mercaptoethanol (0.05 mM, Sigma, St. Louis, MO), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), HEPES buffer (10 mM), and sodium pyruvate (1 mM) was used as culture medium (cRPMI). For dendritic cells isolations, RPMI 1640 Dutch modification (Gibco, Life Technologies, Irvine, Scotland) was used with glutamine, FCS, penicillin, and streptomycin as described above.

Recombinant mouse cytokines were IGIF (produced by expression in Escherichia coli); IL-2 and IL-4 (DNAX); TNFα (Genzyme, Cambridge, MA); and IL-1α (a kind gift from P. Lomedico, Roche, Nutley, NJ). Recombinant mouse IL-12 was obtained from Pharmingen (San Diego, CA) (Schoenhaut et al., 1992).

Purified rat anti-mouse IL-4, 11B11 (Ohara and Paul, 1985), and isotype-matched control immunoglobulins (Abrams, 1995) were supplied by J. Abrams (DNAX). The hybridomas secreting anti-IL-12 MAbs (C17.8.20) were a kind gift from Giorgio Trinchieri (Ozmen et al., 1994). MAbs used for flow cytometry included anti-mouse Mac-1 (M1/70, Caltag, San Francisco, CA) and hamster anti-mouse N418, ATCC (Metlay et al., 1990), and isotype controls. For enrichment of $CD4^+$T cells by magnetic activated cell sorting, biotinylated anti-mouse CD8a, B220, GR-1, Mac-1, and either anti $I\text{-}A^d$ (BALB/c) or $I\text{-}A^b$ (C57BL/6) (Pharmingen) were used. Enriched populations were then stained with anti-mouse CD4 fluorescein isothiocyanate and anti-Mel-14 phycoerythrin (Pharmingen).

For cultures, azide-free, low-endotoxin anti-CD3ε was obtained from Pharmingen. Additional MAbs for ELISA, including anti-IL-4 and anti-IFNγ, were as previously described (Abrams, 1995; Abrams et al., 1992; Macatonia et al., 1993).

The antigenic peptide from chicken ovalbumin ($OVA_{323–339}$) and hen egg lysozyme ($HEL_{74–88}$) were synthesized on an Applied Biosystems (Foster City, CA) model 430 peptide synthesizer.

Preparation of T Cells and APC

$CD4^+$ T cells were enriched by negative selection using magnetic activated cell sorting with a cocktail of biotinylated anti-CD8a, anti-$I\text{-}A^d$, anti-B220, anti-GR1, and anti-Mac-1 antibodies, followed by streptavidin-conjugated microbeads (Miltenyi, Sunnyvale, CA). Enriched $CD4^+$T cells were then further purified using a $FACStar^{Plus}$ flow cytometer (Becton Dickinson) to achieve more than 99% naive $CD4^+$ T cells on the basis of bright Mel-14, $CD4^+$ staining (Bradley et al., 1991). Staining did not alter the function of the T cells (data not shown).

Dendritic cells were enriched from either BALB/c or C57BL/6 spleen cell preparations as described previously (Macatonia et al., 1989, 1993) by first removing adherent cells by overnight culture in plastic flasks, followed by overlayering onto 2 ml metrizamide gradients (analytical grade, 13.7%, Nycomed Pharma AS, Oslo, Norway) and centrifugation for 10 min at 600 × g to yield a population enriched for dendritic cells in the low-density fraction. Dendritic cells ($N418^{hi}Mac\text{-}1^{lo}$) were further purified to homogeneity by flow cytometry as previously described (Macatonia et al., 1993).

Stimulation of Transgenic $CD4^+$T Cells for Cytokine Production

Primary stimulations of $CD4^+$T cells (2.5×10^5/well) were carried out using OVA (0.6 μM) or HEL (1 μM) and irradiated dendritic cells (1×10^4/well, 1000 rad) or red blood cell–lysed spleen cells (5×10^6/well, 3000 rads) as APC in a total volume of 2 ml in 24-well plates. In some cases, antigen and APC were replaced by cross-linked anti-CD3 (10 μg/ml) plus IL-2 (10 ng/ml), when 5×10^5 cells were used per 2 ml well in 24-well plates. Some cultures also received IGIF at 30 ng/ml or IL-12 (10 ng/ml), either separately or in combination. This concentration of IGIF gave optimal proliferation and cyokine production from a Th1 clone (data not shown). In addition, some cultures received 11B11 MAb to block endogenous IL-4 (10 μg/ml). T cells were expanded 3-fold into fresh medium at 72 hr. Cells were harvested on day 7, washed three times, counted, and restimulated with fresh APC (splenocytes or dendritic cells) and 0.6 μM OVA or 1 μM HEL. Cultures previously stimulated with anti-CD3 plus IL-2 were restimulated with anti-CD3 plus IL-2. Cell concentrations were as described for primary stimulation. Some cultures also received IGIF (30 ng/ml) or IL-12 (10 ng/ml), either separately or in combination during the restimulation. Supernatants were collected at 48 hr for measurement of IL-4 and IFNγ.

Committed Th1/Th2 Cells and Th1 Clones

Committed Th1 and Th2 cells were derived from naive TCR transgenic $CD4^+$ T cells as previously described (Murphy et al., 1996). In brief, cells were cultured with antigen ($OVA_{323–339}$ or $HEL_{74–88}$) and splenic APC in the presence of IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) to induce Th1 development or in the presence of IL-4 (10 ng/ml) and anti-IL-12 (10 μg/ml) to produce a Th2 population. Cells were harvested at 7 days and restimulated with fresh APC and antigen in the presence of polarizing cytokines, and this process was repeated for a third cycle. By three rounds of stimulation, T cells achieve a stable, committed Th1 or Th2 phenotype (Murphy et al., 1996).

The Th1 clone HDK1 is a keyhole limpet hemocyanin–specific clone from a BALB/c mouse (Cherwinski et al., 1987).

Both committed Th populations and Th1 clone were used in experiments at least 10 days after their last antigen stimulation and after culture in medium with IL-2 alone. Cells were stimulated in 96-well plates at 5×10^4/well for 48 hr with medium, soluble anti-CD3 (100 ng/ml) and IL-2 (10 ng/ml), or PMA (50 ng/ml) and ionomycin (500 ng/ml). In some cultures IGIF (30 ng/ml), IL-12 (10 ng/ml), and/or IL-1α (10 ng/ml) were added either separately or in combination. Supernatants were collected at 48 hr and assayed for IFNγ and IL-4. Proliferation was assessed by addition of 1 μCi of [^{3}H]thymidine per well for 6 hr.

Cytokine Assays

IFNγ was detected using a two-site sandwich ELISA (Slade and Langhorne, 1989; Abrams, 1995), and sensitivity was 125 pg/ml (1 unit/ml = 0.1 ng/ml). The ELISA for IL-4 has been described previously (Abrams, 1995) with a level of detectability at 150 pg/ml.

Preparation of Nuclear and Whole-Cell Lysates

Prior to the preparation of cell lysates, $1–2 \times 10^7$ Th1 or Th2 cells were incubated in cRPMI with 2% FCS for 5 hr and then cultured at 1×10^6/ml for 30 min in prewarmed medium containing cytokines at 37°C for nuclear lysates and 0, 5, 10, and 30 min for whole-cell lysates. Cytokine concentrations were IGIF 30 ng/ml, IL-1α 10 ng/ml, and IL-12 10 ng/ml. PMA and ionomycin were used at 50 and 500 ng/ml, respectively. Nuclear extracts were prepared as described (Mui et al., 1995). In brief, cells were pelleted and cell membranes then disrupted by brief (1 min) incubation in PSB buffer (50 mM HEPES, 100 mM NaF, 10 mM NaPPi, 2 mM $NaVO_4$, and 4 mM EDTA [all from Sigma]) with 0.2% Nonidet P-40 (NP-40) (Boehringer Mannheim, Indianapolis, IN) and 10 mM $MgCl_2$. Cells were then washed in 0.05% NP-40 with sucrose and 10 mM $MgCl_2$ and nuclei lysed in 0.1% NP-40 in PSB. Protease inhbitors (leupeptin, aprotinin [Sigma], and Pefabloc [Boehringer Mannheim]) were added to all solution used. Whole-cell lysates were prepared in PSB with 0.5% NP-40.

Immunoprecipitation and Western Blot Analysis

Rabbit antiserum to IRAK was a kind gift from Zhaodan Cao, Tularik (South San Francisco, CA). (Cao et al., 1996). One microliter was added to 1 ml of whole-cell lysate and incubated for 2 hr, after which protein A beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added for an additional 2 hr. Beads were then boiled in sodium dodecyl sulfate (SDS) buffer for 3 min and proteins separated by SDS polyacrylamide gel electrophoresis (Novex, San Diego, CA). Separated proteins were transfered to a PVDF membrane (Millipore, Bedford MA), and then Western blotting was performed with antiserum to IRAK at 1:1000 dilution overnight. Western blots were developed with anti-rabbit antibody–horseradish peroxidase (Amersham, Arlington Heights, IL) and chemiluminesence substrate (Pierce, Rockford, IL).

Oligonucleotide Probes and Antibodies for Supershift Assay

Double-stranded oligonucleotide probes used in EMSA were m67SIE, CATTTCCCGTAAATC (sense sequence, no overhang); and NFκB consensus, gatcCAACGCAGGGGAATCTCCCTCTCCTTA (sense sequence, with overhang shown in lower case). Probes were end-labeled with [^{32}P]ATP. The anti-STAT4 MAb NB34 was a kind gift from Nils Jacobson, Washington University (St. Louis, MO) (Guler et al., 1997). Antibodies to Rel family members cRel, RelB, p50, p52, and p65 (RelA) were from Santa Cruz Biotechnology.

EMSA

EMSA was performed as previously described (Mui et al., 1995). Nuclear extracts (2 μg of protein) were incubated with 1 pmol ^{32}P-labeled oligonucleotide probe in binding buffer and 1 μg of poly-dIdC (Pharmingen) for 15 min at room temperature and then run on 5.25% polyacrylamide gels in 0.25× Tris-borate-EDTA. In some cases an excess of unlabeled oligonucleotide probe (100×) was added to lysates for 10 min before the addition of labeled probe. Antibody supershifts were performed by preincubating with 1 μg of NB34 in 2 μl to identify STAT4, or 2 μl of anti-Rel family antibodies to identify NFκB components, for 15 min before the addition of oligonucleotide probes.

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